FEBS 27017

FEBS Letters 537 (2003) 193–197

Preproinsulin mRNA engineering and its application to the regulation of insulin secretion from human hepatomas

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Received 4 November 2002; revised 23 January 2003; accepted 27 January 2003

First published online 7 February 2003

Edited by Robert Barouki

Abstract Cell-based therapies for treating insulin-dependent diabetes (IDD) can provide a more physiologic regulation of blood glucose levels in a less invasive fashion than daily insulin injections. Promising cells include non-β cells genetically engineered to secrete insulin in response to physiologic cues; responsiveness can be introduced at the transcriptional level to regulate preproinsulin (PPI) mRNA biosynthesis. However, these cells exhibit sluggish secretion dynamics, which is not appropriate for achieving euglycemia in higher animals and, eventually, humans. In this work, we have engineered the PPI mRNA so as to destabilize it through nonsense-mediated mRNA decay (NMD). When expressed under transcriptional regulation in HepG2 hepatomas, the engineered PPI mRNA level and of the insulin secretion rate declined faster upon switching off transcription, compared to the one-copy non-engineered control. Our work provides a simple and straightforward method to improve the dynamics of transcriptionally regulated insulin secretion, which can be a useful tool in developing cell-based therapies for IDD.

Key words: Hepatoma; Insulin secretion; Nonsense mRNA; Pancreatic substitute

1. Introduction

Genetic engineering of non-β cells for glucose-responsive insulin secretion offers significant promise in developing a cell-based therapy for insulin-dependent diabetes (IDD). Responsiveness to physiologic stimuli is introduced at the gene transcription level by using promoters up-regulated by glucose and possibly down-regulated by insulin [1–5]. Based on this concept, glucose-regulated insulin expression has been achieved in streptozotocin (STZ)-induced diabetic rodents [2–5]. Although these results are promising, the sluggishness in secretion dynamics of transcriptionally controlled cells makes them inappropriate for glycemic regulation in higher animals and, eventually, humans. Of particular significance is the slow dynamics of secretion down-regulation, which results in the cells secreting insulin long after the stimulus has been removed and may thus revert diabetes to hyperinsulinemia and hypoglycemia, a serious pathological condition.

It has been suggested that the prolonged stability of preproinsulin (PPI) mRNA causes the sluggishness of secretion down-regulation [6,7]. Although prior reports on the PPI mRNA half-life in non-β cells are limited, data from normal and transformed β cells strongly indicate that the stability of PPI mRNA is a limiting factor in expediting secretion down-regulation. In isolated primary rat islets, the half-life of PPI mRNA was estimated to be 77 h under high glucose (17 mM) and 29 h under low glucose concentration (3.3 mM) [8]. In βTC-3 insulinomas, there was only marginal PPI mRNA degradation over 24 h after transcription was stopped [9], while the half-life of PPI mRNA in RIN-5F insulinomas was found to be 58 and 26 h under high (20 mM) and low (3 mM) glucose concentration, respectively [8].

Recently, nonsense-mediated mRNA decay (NMD) has received significant attention because of its biological and medical importance [10–13]. Mutant mRNAs with premature stop codons can be detected by cells via a surveillance mechanism, and are subjected to NMD [14,15]. Although NMD has probably evolved to eliminate erratic mRNAs, it also offers an interesting approach to destabilize genetically engineered mRNA.

In this work, we investigated the destabilization of PPI mRNA through engineering NMD, and its effect on the dynamics of insulin secretion from recombinant HepG2 hepatomas. The implications of our findings in producing appropriately secreting non-β cells with transcriptional regulation of insulin expression are discussed.

2. Materials and methods

2.1. Plasmids for insulin expression

Vectors A–E in Fig. 1 are five plasmids constructed to systematically increase insulin expression in HepG2 cells. Human PPI cDNA wild-type, furin-compatible with furin cleavage sites at the B–C and C–A junctions, and furin-compatible with His B10-to-Asp mutation (B10 mutation) were all generous gifts from Genentech (San Francisco, CA, USA) [16]. The backbone of insulin expression plasmids originated from an 1822 bp *Bgl*II/*Bam*HI fragment of plasmid pRL/Null (Promega, Madison, WI, USA). A 508 bp *Xba*I/*Bam*HI restriction fragment containing both the simian virus 40 (SV40) late polyadenylation and SV40 enhancer signals from plasmid pGL3-control (Promega) was used to replace the SV40 late polyadenylation signal in pRL/Null to prepare the plasmid with the SV40 enhancer. The human cytomegalovirus (CMV) promoter used to drive insulin gene was obtained from an 883 bp *Bgl*II/*Nhe*I restriction fragment of plasmid pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA). The CMV promoter was connected to the *Bgl*II and *Spe*I sites of pRL/Null to prepare the plasmid with an intron, or to the *Bgl*II and *Nhe*I sites to prepare the plasmid without an intron. Different versions of *Xba*I/*Xba*I PPI

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Abbreviations: DOX, doxycycline; IDD, insulin-dependent diabetes; NMD, nonsense-mediated mRNA decay; PPI, preproinsulin

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00121-2

cDNA fragment were connected to the backbones using *Nhe*I and *Xba*I sites.

The backbone of the tet-responsive insulin expression plasmid was obtained from a 2074 bp *Xho*I/*Xba*I fragment of plasmid pRL/Null (Promega). The tetracycline-responsive promoter was obtained from a 448 bp *Xho*I/*Eco*RI restriction fragment of plasmid pTRE2pur (Clontech, Palo Alto, CA, USA). This promoter was inserted into the multiple cloning sites of pRL/Null. For the control plasmid containing one copy of insulin gene, a 360 bp *Xba*I/*Xba*I restriction fragment containing the furin-compatible insulin gene with B10 mutation was connected to the backbone using *Nhe*I and *Xba*I sites (vector F). For the engineered plasmid, two additional copies of the insulin gene were consecutively inserted to the backbone via the *Xba*I site with the same head–tail configuration (vector G). After transcription, the engineered PPI mRNA would contain three consecutive copies of the insulin gene with stop codons in the middle of the transcript. Translation is expected to stop in the middle of the engineered PPI mRNA and induce NMD. The *Xho*I/*Xho*I restriction fragments of the Clontech plasmids cat# K1620-A and K1621-A were used to prepare plasmids pTet-Off and pTet-On, respectively.

2.2. Cell culture and transfection

HepG2 human hepatoma cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1.1 mg/ml sodium pyruvate and 100 units/ml penicillin/streptomycin at 37°C in a 5% CO_2/95% air humidified atmosphere. Approximately 10^6 cells were seeded in each 9.6 cm^2 well of 6-well plates and fed with 2 ml culture medium for 8–12 h prior to transfection. All transfections were carried out with FUGENE 6 reagent (Roche, Indianapolis, IN, USA) following the manufacturer's directions. For each well, HepG2 cells were transfected for 24 h with the plasmid DNA cocktail (2.1 μg or 2.25 μg) and 6 μl of FUGENE 6 reagent. Unless otherwise specified, transfected cells were incubated in culture medium for another 24–36 h for recovery. Cells were then washed and incubated with culture medium for two consecutive 1-h periods to stabilize insulin secretion before any tests.

2.3. Systematic increase of insulin expression

HepG2 cells were transiently transfected using 2 μg of test plasmid (Fig. 1, vector A, B, C, D or E) and 0.1 μg of plasmid pGL3-control (Promega) for luciferase expression as internal standard according to the procedure described in Section 2.2. Cells were then incubated for 1 h in culture medium, which was collected to assay for secreted insulin. Cells were detached using 2.5 ml 0.25% trypsin solution supplemented with 5 mg of collagenase type II for luciferase assay and cell counting.

2.4. Dynamic insulin expression

HepG2 cells were transiently co-transfected using 2 μg of plasmid pTet-Off (or pTet-On) and 0.25 μg of the control or engineered PPI mRNA expression plasmid (Fig. 1, vectors F, G). Tet-Off transfected cells in parallel cultures were incubated in doxycycline (DOX)-free medium or in medium with 1 μg/ml DOX for 24–36 h. Media were renewed, and cells were incubated for an additional 1 h during which insulin secretion was measured; cells were then lysed for quantitative mRNA assay. To study the dynamics of insulin gene down-regulation, Tet-Off transfected cells were switched from DOX-free medium to 1 μg/ml DOX medium for 8 h. Every hour, culture medium was renewed and collected for insulin assay. Every 2 h, cells were lysed for quantitative mRNA assay. To dynamically regulate insulin secretion using the Tet-On system, Tet-On transfected cells were switched from DOX-free medium to 1 μg/ml DOX medium for 1 h, washed four times, then incubated with DOX-free medium for 7 h, and the steps were repeated. Every hour, culture medium was renewed and collected for insulin assay.

2.5. Translation inhibition test

Tet-Off transfected HepG2 cells were washed once, then exposed to DOX-free medium with 28 μg/ml cycloheximide for 4 h [17]. The time of addition of cycloheximide-containing medium was time 0. To resume translation, cells were washed four times, then incubated with DOX-free, cycloheximide-free medium for two consecutive 4-h periods. At 0, 4, 8, and 12 h, cells were lysed for quantitative mRNA assay.

2.6. Assays

Firefly luciferase activity from the internal control vector pGL3-control was measured using the luciferase assay system (Promega) following the manufacturer's protocol and using an LS 5000 scintillation counter (Beckman, Fullerton, CA, USA). Secreted insulin was measured by human insulin specific radioimmunoassay (RIA) kit (LINCO Research, St. Charles, MI, USA). The primary antibody in the kit cross-reacts with proinsulin at less than 0.2%. Radioactivities were determined in Auto-Gamma Counting System, Cobra II (Packard, Meriden, CT, USA). The change of intracellular PPI mRNA level was quantified by the TaqMan real-time polymerase chain reaction (PCR) technique (Applied Biosystems, Foster City, CA, USA). Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA); cDNA was then synthesized using avian myoblastosis virus (AMV) reverse transcriptase kit (Promega) to prepare templates for TaqMan real-time PCR. The expression levels of PPI mRNA and of the internal reference tTA (tetracycline-responsive transcriptional activator, from co-transfected plasmid pTet-Off) mRNA were measured using probes labeled with 6FAM™ and VIC™ (Applied Biosystems), respectively, in separate tubes. The primers and probes (Table 1) were designed using Primer Express software (Applied Biosystems). Measuring PPI mRNA-FAM and tTA mRNA-VIC permitted correction of discrepancies from differences in sample preparation and transfection efficiencies. PCRs were performed with the TaqMan Universal PCR Master Mix and the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the following thermal cycle routine: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. A comparative threshold cycle (C_T) method (User Bulletin Number 2; Applied Biosystems) was used to determine relative gene expression. Relative quantification of PPI mRNA was done by normalizing the PPI mRNA with the tTA mRNA signal and by assigning a value of 100 to the normalized sample level at time zero.

3. Results

3.1. Increase of insulin expression from transfected HepG2 cells

Fig. 1 shows the plasmids constructed to improve insulin expression. With the CMV promoter, vector D resulted in insulin secretion rates of 720 ± 130 fmol/(h·10^6 cells) from transiently transfected HepG2 hepatomas (Fig. 2). This was 32.0 ± 9.4-fold higher than the secretion rate achieved with the furin-compatible insulin gene without any gene attachments (Figs. 1 and 2, vector A), and 27.5 ± 8.0-fold higher relative to the wild-type insulin gene with the chimeric intron and SV40 enhancer (Figs. 1 and 2, vector E). However, because the SV40 enhancer is not compatible with the tet-responsive system, vectors applied for tet-responsive insulin expression did not contain this sequence, but they did contain the B–C and

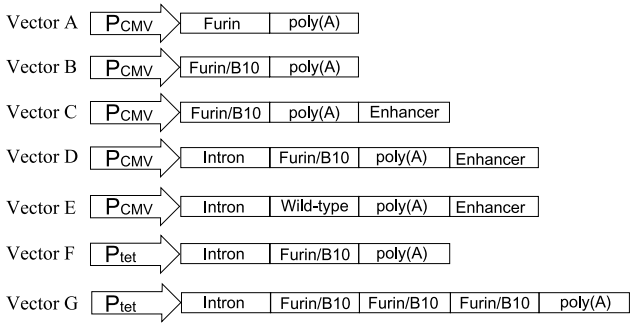

Fig. 1. Plasmid structures. Vectors A, B, C, D and E were constructed for evaluating elements that increase insulin expression. Vector F and G are the tet-responsive plasmids with one and three copies, respectively, of PPI cDNA.

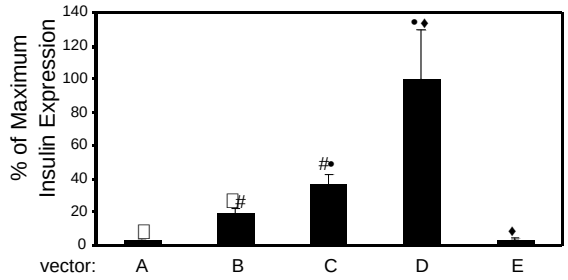

Fig. 2. Systematic increase of insulin expression from transfected HepG2 hepatomas. Cells were transiently co-transfected with the test vector shown in Fig. 1 (A, B, C, D or E) and the internal control plasmid pGL3-control. Luciferase, expressed through pGL3-control, was used to normalize the insulin secretion rate and thus correct for variations in transfection efficiency. The normalized insulin secretion rate from vector D (highest insulin expression vector) was assigned a value of 100. Experiments were performed in triplicate wells. *, # and ♦ indicate P values <0.02, and • indicates P value <0.04. P values were calculated using a one-tailed t-test, assuming unequal variances. Bars indicate standard deviation.

C–A junctions and B10 His-to-Asp mutations, as well as the chimeric intron.

3.2. PPI mRNA engineering and expression

The control and engineered PPI mRNA expression plasmids (vectors F and G, Fig. 1) were each transiently co-transfected with plasmid pTet-Off in HepG2 cells. Use of the engineered PPI mRNA decreased the insulin expression level to 109 ± 43 fmol/(h·10^6 cells) from 320 ± 55 fmol/(h 10^6 cells) ($n = 4$) of the control construct, or by 66%. Evidently, the decreased stability of engineered mRNA reduced intracellular mRNA levels and thus expression, as observed in many other nonsense mutations [14,15]. However, cells with both engineered and control plasmids were regulated in a similar fashion by DOX. With the control plasmid, transfected cells had a 24.8 ± 5.2-fold higher mRNA expression and a 20.7 ± 3.8-fold higher insulin secretion rate in the absence relative to the presence of DOX. With the engineered plasmid, the corresponding values were 20.7 ± 3.9-fold higher mRNA and 19.2 ± 4.8-fold higher insulin secretion rate in the absence vs. presence of DOX.

3.3. Dynamics of down-regulation of insulin expression

The down-regulation of insulin gene expression was tested using the Tet-Off expression system. In the dynamic test, transcription was down-regulated by exposing cells at time zero to culture medium with 1 μg/ml DOX (Fig. 3). The engineered PPI mRNA exhibited a faster decline with a half-life of less than 4 h relative to the control which had a half-life of more than 8 h. The decline of insulin secretion rate followed the similar trends. Thus, immunoreactive insulin can be synthesized from the engineered PPI mRNA, and the dynamics of down-regulation upon switching off transcription at both the mRNA and secreted protein levels are faster with the engineered PPI mRNA relative to control.

Table 1
Probe and primer sequences for TaqMan real-time PCR technique

Gene	Probe and primer sequence (5′→3′)
Insulin	FAM probe: TCCGACCTGGTGGAAGCTCTCTACCTAGTG
	forward: TTTGTGAACCAACACCTGTGC
	reverse: GGGTGTGTAGAAGAAGCCTCGTT
tTA	VIC probe: CCCGTAAACTCGCCCAGAAGCTAGGTGT
	forward: GGTCGGAATCGAAGGTTTAACA
	reverse: TGCCAATACAATGTAGGCTGCT

3.4. Regulation of insulin secretion using the Tet-On system

Up- and down-regulation of insulin secretion from transfected HepG2 cells was tested using the Tet-On system, in which DOX induces insulin gene expression. Using 1 h of induction in an 8-h cycle, insulin production from the engineered PPI mRNA responded to the transcriptional switches (Fig. 4). On the other hand, insulin production from the control PPI mRNA failed to decline during the 7-h basal periods of exposure to DOX-free medium.

3.5. Translation inhibition test

A control experiment was performed to elucidate whether NMD was indeed involved in shortening the half-life of the engineered PPI mRNA. Since normal mRNA and nonsense mutants are distinguished from each other via translation, the effect of inhibiting translation on the levels of control and engineered PPI mRNA was examined. HepG2 hepatomas transiently transfected with control or engineered PPI mRNA under Tet-Off control were maintained in DOX-free medium and exposed to cycloheximide at time 0 (Fig. 5). The engineered/control PPI mRNA ratio at time 0 was defined as 100%. After 4 h of cycloheximide treatment, this ratio increased to 490% compared to the cycloheximide-free culture. Upon withdrawing cycloheximide to resume translation, the engineered/control PPI mRNA ratio decreased toward the basal level.

4. Discussion

Cell sourcing constitutes a critical issue in developing a cell-based therapy for treatment of IDD. Non-β cells, primarily cells of hepatic origin, are considered by several research groups as hosts for recombinant insulin expression under transcriptional regulation [1–5]. A major advantage of these cells is that they are potentially autologous, retrieved as a

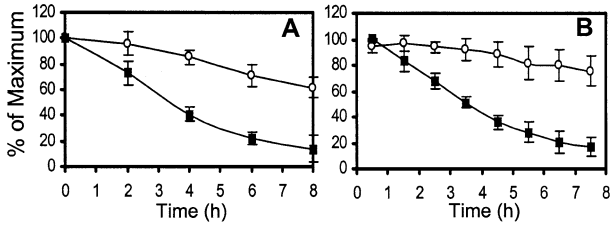

Fig. 3. Dynamics of down-regulation of insulin gene expression using Tet-Off system with control (open circles) and engineered (filled squares) PPI mRNA expression. A: Down-regulation of PPI mRNA. B: Down-regulation of insulin secretion rate. tTA mRNA was used as an internal standard for quantitative PPI mRNA assay. In each independent test, the PPI mRNA was normalized by designating the sample without DOX treatment ($t = 0$) as the calibrator and setting it at 100%; insulin secretion rates were normalized by designating the sample with the highest rate as the calibrator and setting it at 100%. After 8-h down-regulation, the control and the engineered PPI mRNA decreased to $61 \pm 8\%$ ($P < 0.007$) and $14 \pm 10\%$ ($P < 0.003$), respectively, and the insulin secretion rates to $75 \pm 12\%$ ($P < 0.05$) and $17 \pm 7\%$ ($P < 0.002$), respectively. Each experiment involved three independent tests. Bars indicate standard deviations.

biopsy from the patient. A disadvantage is that transcriptionally controlled cells exhibit sluggish secretion dynamics and thus may not be suitable, as such, for achieving normoglycemia in higher diabetic animals and humans. To expedite the dynamics of secretion down-regulation, translation needs to stop soon after transcription has been turned off.

The topic of modulation of mRNA stability is currently under intense investigation [18–20]. Specifically with PPI mRNA, to accelerate the rate of mRNA turnover, the use of antisense RNA [21] or connecting the insulin gene with the 3′-untranslated region (3′-UTR) of some labile mRNAs, such as those encoding cytokines, have been considered [7]. Compared to these, our approach involving NMD is simple and straightforward. It is also expected to be generic and applicable to different host cells, including primary cells.

NMD improved the secretory response of HepG2 cells but also reduced intracellular PPI mRNA levels and thus insulin expression. To ameliorate this problem, we increased insulin expression before applying NMD. HepG2 hepatomas transiently transfected with vector D (Fig. 1) secreted insulin at a rate of 720 fmol/(h·10^6 cells), which is higher than the basal insulin secretion rate of 380 fmol/(h·10^6 cells) exhibited by βTC-3 mouse insulinomas [22]. For tet-responsive insulin expression, although the SV40 enhancer was not applied, the insulin secretion from Tet-Off transfected HepG2 in DOX-free medium was 320 fmol/(h·10^6 cells) and 109 fmol/(h·10^6 cells) when the control and engineered PPI mRNA, respectively, were used.

The specificity of the Tet-Off system and the sub-toxic DOX concentration used in this study [23] increase the fidelity of mRNA half-life measurements compared to experiments with non-specific inhibitors [24]. One concern in determining insulin secretion dynamics with the tet-responsive system is the pharmacokinetics of DOX. It has been shown that the Tet-Off system and its transcription repressor tetracycline can be applied to measure the stability of a spliced intron with a half-life as low as 6 min [25]. Hence, it appears that tetracycline quickly diffuses into cells and blocks transcription. We expect DOX, a tetracycline isomer and analog, to have similar kinetics in regulating insulin gene expression in HepG2. However, the reverse process of decline of intracellular DOX concentration may be more complicated since, besides diffusion out, processes such as dissociation of DOX from the DOX–protein complex, distribution and accumulation of DOX in organelles, and metabolism of DOX may be occurring and contributing to the delays in insulin secretion down-regulation exhibited by the Tet-On system (Fig. 4). It is possible that with a metabolizable secretagogue, e.g. glucose, the intrinsic kinetics of transcription are closer to the kinetics of secretion, especially during the down-regulation process.

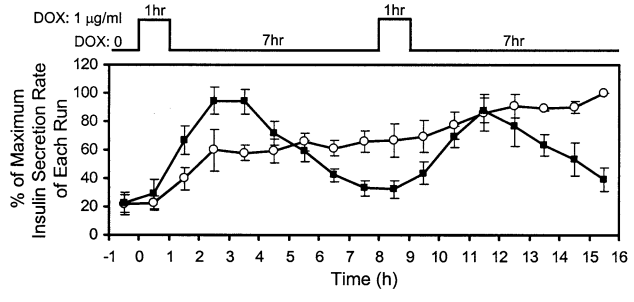

Fig. 4. Regulation of insulin secretion from transfected HepG2 cells using the Tet-On system with control (open circles) and engineered (filled squares) PPI mRNA expression. In each independent test, the insulin secretion rates were normalized by designating the sample with the highest rate as the calibrator and setting it at 100%. Each experiment involved three independent tests. Bars indicate standard deviations.

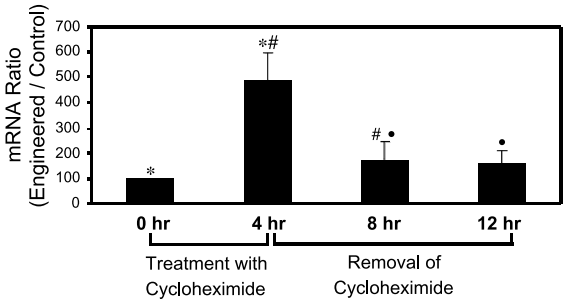

Fig. 5. Effect of translation on PPI mRNA stability (evidence of NMD). HepG2 hepatomas were transiently co-transfected with pTet-Off and the control or engineered PPI mRNA expression plasmid. tTA mRNA was used as an internal standard for quantitative PPI mRNA assay. In each independent test, the PPI mRNA ratios (engineered/control) were normalized against the sample without cycloheximide treatment ($t = 0$), which was set at 100%. Each experiment involved four independent tests. * and # indicate P values < 0.003, and • indicates P value $= 0.36$. Bars indicate standard deviations.

In Fig. 4, we applied two DOX square waves to induce the Tet-On system, and obtained a 5-fold increase of insulin secretion from the single copy plasmid. We have also performed an 8-h induction with the same Tet-On system, and obtained an approximately 30-fold increase of insulin secretion (data not shown). This result is similar to the findings of Gossen et al. [23] using luciferase as the reporter to test the kinetics of the Tet-On system. Due to the stability of the one-copy PPI mRNA plasmid, there was no decline in insulin secretion rate during the two 7 h DOX-free basal periods. However, the increase in insulin secretion from the second induction was significantly lower than the first. If the experiment continued in the same fashion, we would expect the insulin secretion to not exhibit any net increase after some maximum, as any (small) additional increase in secretion rate upon induction would be compensated by a decline during the subsequent basal period.

This work constitutes a first step towards developing potentially autologous, genetically engineered, transcriptionally controlled non-β cells for treatment of IDD. Clearly, the NMD-mediated improvement of secretion dynamics will need to be validated in primary cells expressing insulin under a glucose-responsive promoter. In this, it is important that the host cells and the insulin gene be of the same species, as species mismatch may alter the stability of PPI mRNA considerably. It should be noted, however, that there exists no evidence that the NMD methodology would not be applicable to primary cells. Finally, the recombinant cells will need to be studied in an in vivo situation, as the insulin secretion dynamics might be different in culture and in vivo.

Acknowledgements: This work was supported primarily by the ERC Program of the National Science Foundation under Award Number EEC-9731643. We thank Genentech, Inc. for supplying the wild-type and modified human PPI cDNA.

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